Free Radical Biology & Medicine 51 (2011) 1575–1582

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage

Filipe Cabreiro [a,1], Daniel Ackerman [a,1], Ryan Doonan [a,2], Caroline Araiz [a], Patricia Back [b], Diana Papp [a,c], Bart P. Braeckman [b], David Gems [a,*]

[a] *Institute of Healthy Ageing and Research Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, UK*
[b] *Laboratory for Aging Physiology and Molecular Evolution, Department of Biology, Ghent University, Ghent, Belgium*
[c] *Department of Medical Chemistry, Semmelweis University, 1094 Budapest, Hungary*

ARTICLE INFO

Article history:
Received 17 April 2011
Revised 30 June 2011
Accepted 24 July 2011
Available online 31 July 2011

Keywords:
Aging
Caenorhabditis elegans
daf-16/FoxO
ER stress
Oxidative damage
Superoxide dismutase
Free radicals

ABSTRACT

The superoxide free radical ($O_2^{\bullet-}$) has been viewed as a likely major contributor to aging. If this is correct, then superoxide dismutase (SOD), which removes $O_2^{\bullet-}$, should contribute to longevity assurance. In *Caenorhabditis elegans*, overexpression (OE) of the major cytosolic Cu/Zn-SOD, *sod-1*, increases life span. But is this increase caused by enhanced antioxidant defense? *sod-1* OE did not reduce measures of lipid oxidation or glycation and actually increased levels of protein oxidation. The effect of *sod-1* OE on life span was dependent on the DAF-16/FoxO transcription factor (TF) and, partially, on the heat shock TF HSF-1. Similarly, overexpression of *sod-2* (major mitochondrial Mn-SOD) resulted in life-span extension that was *daf-16* dependent. *sod-1* OE increased steady-state hydrogen peroxide (H_2O_2) levels in vivo. However, co-overexpression of catalase did not suppress the life-span extension, arguing against H_2O_2 as a cause of longevity. *sod-1* OE increased *hsp-4* expression, suggesting increased endoplasmic reticulum (ER) stress. Moreover, longevity was partially suppressed by inactivation of *ire-1* and *xbp-1*, mediators of the ER stress response. This suggests that high levels of SOD-1 protein may challenge protein-folding homeostasis, triggering a *daf-16*- and *hsf-1*-dependent stress response that extends life span. These findings imply that SOD overexpression increases *C. elegans* life span, not by removal of $O_2^{\bullet-}$, but instead by activating longevity-promoting transcription factors.

The oxidative damage theory proposes that accumulation of molecular damage caused by reactive oxygen species (ROS), particularly superoxide ($O_2^{\bullet-}$) and its derivatives, is a primary cause of aging [1,2]. The enzyme superoxide dismutase (SOD) converts $O_2^{\bullet-}$ into hydrogen peroxide (H_2O_2), which in turn is converted into H_2O and O_2 by the action of catalase, glutathione peroxidase, and other enzymes. The oxidative damage theory suggests that SOD should protect against aging as well as oxidative damage. However, recent studies in *Caenorhabditis elegans* have found little support for this prediction [3–5]. For example, administration of SOD mimetic compounds fails to increase life span [5–7] and low concentrations of $O_2^{\bullet-}$ generators actually increase life span [8–10]. Moreover, for four of the five *C. elegans sod* genes, abrogation of function has little or no effect on aging [11–14].

However, the effects of manipulation of expression of *sod-1*, the major cytosolic Cu/Zn-SOD isoform, could imply that cytosolic $O_2^{\bullet-}$ and the damage that it causes contribute to *C. elegans* aging. Abrogation of *sod-1* by gene deletion or RNA-mediated interference (RNAi) slightly reduces life span [11,13–15] and *sod-1* overexpression (OE) can increase life span [11]. However, in that study we did not verify that *sod-1* OE reduces levels of oxidative damage. Moreover, *sod-1* OE lines were hypersensitive rather than resistant to oxidative stress, hinting that SOD OE might not reduce ROS-induced damage in these strains. It is also not known whether SOD levels in wild-type worms are limiting with respected to ROS detoxification.

The effect on life span of transgene-induced elevation of SOD has been tested in several model organisms, with mixed results (reviewed by [16]). Like its effect in *C. elegans*, SOD OE can extend life span in *Drosophila melanogaster*[17,18], but in mice, ubiquitous overexpression of SOD does not extend life span [19]. A potential problem with this approach to testing the oxidative damage theory is that SOD OE can have a pro-oxidant effect, leading to more rather than less oxidative damage [20,21]. For example, elevated levels of SOD can markedly increase H_2O_2 levels in vivo [22]. There are other instances of manipulations of antioxidant defenses having unpredictable effects on redox status and

---

Abbreviations: HNE, 4-hydroxynonenal; AMPK, AMP-dependent kinase; CML, carboxymethyllysine; co-OE, co-overexpression; HSF-1, heat shock factor-1; IIS, insulin/IGF-1 signaling; NAC, *N*-acetylcysteine; OE, overexpression; ROS, reactive oxygen species; RNAi, RNA-mediated interference; $O_2^{\bullet-}$, superoxide anion; SOD, superoxide dismutase.

* Corresponding author. Fax: +44 20 7679 7096.
E-mail address: david.gems@ucl.ac.uk (D. Gems).

[1] These authors contributed equally to this work.

[2] Current address: Department of Biological Sciences, City University of New York, New York, NY, USA.

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.07.020

damage levels. In worms, loss of the peroxiredoxin *prdx-2* leads to up-regulation of genes involved in phase II detoxification [23]. In mice, loss of glutathione peroxidase can lead to health benefits through altered insulin signaling [24]. In humans, the beneficial effects of physical exercise can be blocked by consumption of antioxidants, implying that ROS have a positive role in conferring these health benefits [25].

All this raises doubts as to whether overexpression of *sod-1* in *C. elegans* extends life span by reducing levels of oxidative damage. Given that SOD OE can increase levels of H_2O_2 and of damage in some contexts, one possibility is that this occurs in worms and increases life span via hormesis. In this phenomenon, exposure to sublethal stress induces an adaptive response resulting in beneficial effects that include increased life span [26]. In *C. elegans,* life span can be increased by transient exposure to stressors, such as heat and hyperoxia, or chronic exposure to low levels of chemical ROS generators [8,10,27–29]. Hormetic effects on life span can require the FoxO transcription factor DAF-16 [8,30], which is also required for life-span extension by reduced insulin/IGF-1 signaling (IIS) [31]. This suggests that activation of DAF-16 can mediate hormetic effects, perhaps via mechanisms similar to those operative in long-lived IIS mutants.

If *sod-1* OE extends life span by reducing ROS levels, one would expect to see an associated reduction in levels of oxidative damage. However, if a hormetic effect were occurring, then one would not necessarily expect to see reduced oxidative damage. Moreover, the effect on longevity might require mediators of the stress response, such as DAF-16. In this study we investigate these possibilities. Our results support the second scenario and strongly imply that the longevity of *sod-1* OE lines is not attributable to enhanced antioxidant defense. This means that the longevity of *sod-1* OE lines is not evidence of the veracity of the oxidative damage theory of aging.

Materials and methods

Strains and culture conditions

Nematode strains used in this study included the following: DR1563, *daf-2(e1370);* GA114, *daf-16(mgDf50); daf-2(e1370);* GA184, *sod-2 (gk257);* GA222, *wuEx118* [*sod-2* gDNA *rol-6(su1006)*]; GA226, *wuEx122* [*sod-1* gDNA *rol-6(su1006)*]; GA228, *wuEx123* [*sod-1* gDNA *rol-6(su1006)*]; GA230, *wuEx125* [*sod-1* gDNA *rol-6(su1006)*]; GA644, *wuEx123* [*sod-1* gDNA *rol-6(su1006)*]; *daf-16(mgDf50);* GA646, *wuIs152* [*sod-1* gDNA *rol-6(su1006)*] *hsf-1(sy441);* GA648, *wuIs152* [*sod-1 gDNA rol-6(su1006)*] *aak-2(ok524);* GA697, *zcIs4* [*hsp-4::gfp*] *wuEx196* [*rol-6 (su1006)*]; GA800, *wuIs151* [*ctl-1, -2, -3 gDNA myo-2::gfp*]; GA801, *wuIs152* [*sod-1 gDNA rol-6(su1006)*]; GA804, *wuIs155* [*sod-2* gDNA *rol-6 (su1006)*]; GA805, *wuIs156* [*sod-2* gDNA *rol-6(su1006)*]; GA808, *wuIs151* [*ctl-1, -2, -3 gDNA myo-2::gfp*]; *wuIs156* [*sod-2* gDNA, *rol-6(su1006)*]; GA809, *wuIs151* [*ctl-1, -2, -3 gDNA, myo-2::gfp*]; *wuIs152* [*sod-1* gDNA *rol-6(su1006)*]; GA812, *wuIs156* [*sod-2* gDNA *rol-6(su1006)*]; *daf-16 (mgDf50);* GA824, *wuEx196* [*rol-6(su1006)*]; GA1001, *aak-2(ok524)* (derived by outcrossing RB754); GA1407, *zcIs4* [*hsp-4::gfp*] wu*Is152* [*sod-1 gDNA rol-6(su1006)*]; GA1423, *zcIs13* [*hsp-6::gfp*] wu*Is152* [*sod-1 gDNA rol-6(su1006)*]; GA1424, *zcIs13* [*hsp-6::gfp*] wu*Is152* [*sod-1 gDNA rol-6(su1006)*]; GA1425, *zcIs13* [*hsp-6::gfp*] wu*Is152* [*sod-1 gDNA rol-6 (su1006)*]; GA1429, *zcIs13* [*hsp-6::gfp*] *wuEx196* [*rol-6(su1006)*]; GA1430, *zcIs13* [*hsp-6::gfp*] *wuEx196* [*rol-6(su1006)*]; GR1307, *daf-16 (mgDf50)*; and PS3551, *hsf-1(sy441)*. Standard nematode culture conditions were used [32].

Nematode strain construction

To prepare *C. elegans* lines overexpressing *sod-2,* a genomic DNA fragment containing the *sod-2* coding sequence was amplified by PCR. This was injected into worms at 55 ng/μl, creating a line containing the *wuEx118* transgene array. Primers used for PCR were 5′-TGAATCC-TACGGAAAGTGCC-3′ and 5′-TCAATGAATGGACAGGTTTCCC-3′. *wuIs155* and *wuIs156* were created by integration of *wuEx118* using X-irradiation. Integrant lines were outcrossed to N2 six times. Co-overexpression with catalase was achieved by combining the transgene arrays *wuIs155* and *wuIs151* [*ctl-1,-2,-3* overexpression] in a single line. The construction of *wuIs151* and of *wuIs152* [*sod-1* overexpression] has been described previously [11].

Survival analysis

C. elegans hermaphrodites were grown at 20 °C and transferred to plates containing 10 μM fluorodeoxyuridine at the L4 stage of development. Death was scored as described previously [33]. Survivorship of populations was compared statistically using the log-rank and Wilcoxon tests, performed using the application JMP 7.0.1 (SAS).

N-acetylcysteine (NAC) treatment

Worms were transferred to control or NAC plates as L4/young adults and were transferred to freshly prepared plates every 7 days. NAC was added to plates topically from a freshly prepared NAC stock solution.

Use of HyPer fluorescent probe to measure in vivo H_2O_2 levels in C. elegans

HyPer is a genetically encoded H_2O_2-specific fluorescent probe [34]. This yellow fluorescent protein-based probe is both sensitive and specific and the fluorescence measurement is ratiometric and therefore not dependent upon the expression level. *unc-119* mutant worms were transformed by biolistic transformation with a vector containing the wild-type *unc-119* gene, the promoter of the constitutive and ubiquitously expressed *rpl-17* gene, and the *hyper* coding region. Homozygous *hyper* transgenic worms were back-crossed twice into wild-type (N2). *wuIs152* [*sod-1*] was then crossed into the *hyper* strain. Nematodes were cultured on NA agar seeded with *Escherichia coli* K12 as described previously [32]. Transgenic worms expressing *hyper* only (controls) or *hyper* plus *sod-1* OE [*wuIs152*] were raised at 24 °C and harvested 1 day after the L3/L4 molt. Worms were washed with S-buffer (43.55 mM KH_2PO_4, 6.45 mM K_2HPO_4, and 100 mM NaCl in distilled water, pH 6) and next with 2.5 mM EDTA in S-buffer (to eliminate bacteria adhering to the cuticle) and pipetted as a dense pellet of at least 1000 worms in a black, flat-bottomed 96-microtiter plate well (Greiner). The fluorescent signal was measured over a 10-min period using a Victor^2 1420 multilabel counter (PerkinElmer) at 25 °C with the excitation filters FP490 and FP405 and emission filter F535. The data shown represent the average ratios over 10 min of four biological replicates (at least three technical replicates each). A fuller description of the validation of this technique as a measure for standing H_2O_2 levels will be published soon by P. Back et al.

Harvesting nematodes for biochemical assays

Synchronous cultures were initiated via alkaline hypochlorite lysis of gravid adults. Eggs were allowed to hatch overnight in M9 buffer at 20 °C and resulting L1 larvae were grown on NGM plates seeded with *E. coli* OP50. At harvest, late L4 larvae and young adult worms were rinsed off the plates, washed with M9 buffer, and stored at −75 °C until use. For all assays, three to five replicate worm cultures were used.

Preparation of protein extracts via sonication

Worm samples were homogenized using a Bioruptor (Cosmo Bio Co., Ltd, Tokyo, Japan) in 2-ml microcentrifuge tubes containing equal amounts of suspended worms and CelLytic (Sigma) and 1× protease